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The Effects of a Nonimmunogenic Form of Murine Soluble Interferon-γ Receptor on the Development of Autoimmune Diabetes in the NOD Mouse

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ABSTRACT

Previous studies have shown that *in vivo* treatment with antiinterferon-γ (anti-IFNγ) monoclonal antibodies (mAbs) prevents the development of autoimmune diabetes in NOD mice. Although these findings anticipate that specific anti-IFNγ therapies may be useful for the prevention/treatment of human insulin-dependent diabetes mellitus, there are several reasons why the use of anti-IFNγ mAb may be difficult in the clinical setting. With the aim to develop alternative forms of specific anti-IFNγ therapies, we recently produced a nonimmunogenic form of the soluble IFNγ receptor (sIFNγR) that binds and neutralizes murine IFNγ with an affinity higher than that of anti-IFNγ mAb. In this study we compared the efficacy of sIFNγR to that of two anti-IFNγ mAbs (XMG 1.2 and AN-18) in the prevention of spontaneous and accelerated (cyclophosphamide-induced) forms of autoimmune diabetes in NOD mice. The results show that in the spontaneous model, sIFNγR could prevent histological and clinical signs of autoimmune diabetes as efficiently as the two mAbs. Under *ex vivo* conditions, sIFNγR exhibited a more powerful modulatory effect than XMG1.2 mAb on cytokine secretion from splenic lymphoid cells, which resulted in a significant reduction of Concanavalin A-induced IL-2 secretion and an augmented release of both unstimulated and lipopolysaccharide-induced IL-6. Moreover, although both mAbs were immunogenic and elicited formation of high titers of anti-rat IgG, sIFNγR did not induce antibody formation. Unexpectedly, in the cyclophosphamide-induced model, sIFNγR turned out to be less effective than either of the two anti-IFNγ mAbs. Taken together, these data support the role of IFNγ in the pathogenesis of NOD mice, but, more importantly, suggest that a nonimmunogenic approach is possible to the diminution of the effects of IFNγ in this model. (*Endocrinology* 137: 5567–5575, 1996)

INTERFERON-γ (IFNγ) is a cytokine produced by T lymphocytes and natural killer cells that exhibits pleiotropic effects on the immune system (see Ref. 1 for review). Several lines of evidence suggest that IFNγ may favor both systemic and organ-specific autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis, autoimmune thyroid diseases, and type 1 diabetes [insulin-dependent diabetes mellitus (IDDM), autoimmune diabetes]) (see Refs. 1–3 for reviews). In particular, the observation that anti-IFNγ monoclonal antibodies (mAbs) prevent the development of hyperglycemia in animal models of IDDM such as the NOD mouse (4–6), the BB rat (7), and the mouse made diabetic with multiple doses of streptozotocin (8) anticipates that blockade of endogenous IFNγ with specific antagonists may also exert beneficial effects in human IDDM.

To date, in experimental studies conducted in animal models of immunoinflammatory diseases, IFNγ bioactivity has primarily been inhibited by treatment with mAbs directed against the cytokine or its receptor. However, specific anti-IFNγ therapy with mAbs may not be easily feasible in the clinical setting. As observed in transplantation studies with OKT3 mAb, the heterologous origin of the protein often elicits a humoral immune response consisting of antixenotypic and antiidiotypic antibodies capable of reducing the bioactivity of the mAb (9, 10). Attempts to diminish the immunogenicity of mAbs in humans by creating chimeric (mouse-human) or fully humanized mAbs have in some cases been unsuccessful, as these mAbs may still induce the production of both neutralizing and antiidiotype antibodies (11, 12). Theoretically, the prolonged treatment with mAb can also lead to immune complex formation, the deposition of which may further enhance local and systemic immunoinflammatory reactions through activation of the complement pathway.

As IFNγ acts through binding to specific receptors (IFNγR) expressed on the surface of the target cells, an alternative form of specific anti-IFNγ therapy may consist of administering a soluble form of the IFNγR. This would neutralize the endogenous IFNγ, thus abrogating its immunomodulatory effects. For this purpose, we recently produced and characterized a soluble form of the mouse IFNγR (sIFNγR) that is not immunogenic, is capable of neutralizing murine IFNγ bioactivity *in vitro* and *in vivo*, has an affinity higher than that of anti-IFNγ mAbs (13, 14), and prevents SLE-like syndrome in the (NZB×NZW)F1 mouse (15). Importantly, even during prolonged (14 days) administration, sIFNγR does not induce antibody formation in BALB/c mice with doses up to 100 μg/day (14).

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In this study we evaluated the effects of prolonged prophylactic treatment with sIFNγR on the development of spontaneous and accelerated [cyclophosphamide (CY)-induced] forms of autoimmune diabetes in NOD mice, and the results were compared with those obtained using either of the two rat antimouse IFNγ mAbs, XMG 1.2 and AN-18. The data demonstrate that prophylaxis with sIFNγR is as effective as either of the two anti-IFNγ mAbs in preventing spontaneous, but not CY-induced, diabetogenesis in NOD mice. Moreover, although both XMG1.2 and AN-18 provoked the formation of antirat IgG upon prolonged (8 weeks) treatment, no significant rise in the serum titer of anti-sIFNγR antibody was observable in NOD mice treated for the same time with sIFNγR.

Materials and Methods

Mice

Female NOD/Lt mice were provided by Jackson Laboratories (Bar Harbor ME). The mice were kept under standard laboratory conditions (nonspecific pathogen free) with free access to food and water and were cared for according to the guidelines of the local committee for animal research. They were allowed to adapt for at least 1 week to their environment before commencing the experiment. Mice were defined as diabetic on the basis of 2 consecutive days of glycosuria (Tes-tape, Eli Lilly Co., Indianapolis, IN), followed by fasting glycemia above 210 mg/dl. Under these experimental conditions, 60–75% of female NOD/Lt mice develop diabetes between 12-35 weeks of age.

Anti-IFNγ mAbs

The XMG 1.2 mAb is a rat IgG1 anti-mouse IFNγ (16); its neutralizing titer was 10 U mouse IFNγ/4 ng protein as assessed on L929 cells. The rat mAb AN-18 is an IgG2a anti-mouse IFNγ previously produced and characterized by Prat and colleagues (17). The antibody neutralizes the antiviral activity of both natural and recombinant mouse IFNγ, but does not react with murine IFNα or -β. The neutralizing titer of the AN-18 mAb was 10 U mouse IFNγ/12 ng protein, as assessed using L929 cells.

Murine sIFNγR

The extracellular domain of the mouse IFNγR (sIFNγR) was expressed in Sf9 insect cells infected with recombinant baculoviruses. The R was expressed, purified, and biochemically characterized, as described previously (14, 18).

Protein and chemicals

Control rat IgG, Concanavalin A (Con A), and lipopolysaccharide (LPS) were obtained from Sigma Chemical Co. (St. Louis, MO). CY was provided by Schering-Plough (Milan, Italy).

Experimental design

Spontaneous IDDM. Euglycemic female NOD, randomly allocated into different groups, received either anti-IFNγ mAbs or sIFNγR starting at the 4th or the 16th week of age. Because insulitis is absent in 4-week-old NOD mice and is actively ongoing in virtually all animals by the age of 16 weeks (19), this experimental approach allowed us to investigate the role of endogenous IFNγ in both afferent and efferent phases of the diabetogenic process in the NOD mouse.

For the early prophylactic treatment, four groups of 4-week-old female euglycemic NOD mice were created that were treated with PBS (group A), irrelevant rat IgG (group B), XMG1.2 (group C), AN-18 (D), or sIFNγR (group E) according to the doses and treatment schedule described in Fig. 1. Irrelevant rat IgG were used as controls for either XMG 1.2 or AN-18 mAbs, and PBS was used as the control for sIFNγR. Although PBS has been previously employed by ourselves and others as a control for soluble cytokine receptors when these reagents were used in various models of autoimmune diseases (20–22), it is worth mentioning that PBS does not provide a suitable control for the soluble receptor, which should, instead, consist of fragments of the receptor that do not to bind the cytokine *in vitro* or *in vivo*. Because these fragments are not yet available for sIFNγR, we decided to use PBS as control for sIFNγR.

After 9 weeks of continuous treatment, the mice were killed, and their pancreata specimens were collected and examined for the prevalence and severity of insulitis. Mice becoming diabetic before this age were killed and not included in the histological examination.

Moreover, to evaluate the effects of these IFNγ inhibitors on the cytokine secretory capacity of NOD mice, spleens were collected at death from individual mice from groups B, C, and E; passed through a sterile sieve; and suspended in Hanks' Balanced Salt Solution (HBSS). Splenic lymphoid cells (SLC), obtained and cultured for 48 h at 37 C with 5% CO_2, as described previously (23), were either unstimulated or stimulated with Con A or LPS. At the end of the culture period, the cells were centrifuged, and the supernatant collected was aliquoted at −20 C until assayed for the content of interleukin-2 (IL-2), IL-4, IL-6, IL-10, IFNγ, and tumor necrosis factor-α (TNFα). These cytokines were measured using specific mouse-specific solid phase enzyme-linked immunosorbent assay (ELISA), purchased by Biosource (Camarillo, CA), for IL-2, IL-4 IL-6, IL-10, and IFNγ and Endogen (Boston, MA) for TNFα. Undiluted or 2-fold (IL-6) diluted supernatants were run in duplicate and assayed following the manufacturers' instructions. The limits of sensitivity of the assays were 13 pg/ml for IL-2, 5 pg/ml for IL-4, 8 pg/ml for IL-6, 13 pg/ml for IL-10, 1 pg/ml for IFNγ, and 15 pg/ml for TNF-α.

For the late prophylactic treatment, 16-week-old euglycemic female NOD mice were divided into 5 groups (F, G, H, I, and J) and treated with PBS (F), irrelevant rat IgG (G), XMG 1.2 (H), AN-18 (I), or sIFNγR (J) according to the experimental protocol shown in Table 1. Mice were screened for diabetes once a week. The mice from groups F, G, and H were killed either at the onset of diabetes or at the end of the experiment (24 weeks of age), and their pancreata specimens were collected for histological analysis. Diabetic mice from groups I and J were also killed at the onset of the disease; in contrast, only 4 of 9 and 7 of 17 of the remaining euglycemic animals from the sIFNγR- and AN-18-treated groups were killed at the end of the study for histological examination of the β-cells, and their SLC were used for the IDDM transfer experiment (see after). After treatment withdrawal, the remaining euglycemic mice were kept for a 3-month follow-up period to determine whether sIFNγR or AN-18 prophylaxis afforded temporary or permanent protection from the disease.

CY-induced IDDM. Another set of experiments was performed to evaluate the effects of sIFNγR on the accelerated model of diabetes that can be provoked in NOD mice with CY. For this purpose, seven groups of 100- to 120-day-old female NOD mice were studied; the animals were injected with CY and treated according to the experimental design shown in Table 2. Mice were screened for the development of diabetes on days 14 and 15 after CY challenge. On day 15, all mice from these groups were killed, and their pancreata specimens were analyzed to evaluate the extent and severity of the insulitis process.

IDDM transfer studies. Finally, we wondered whether prolonged prophylactic treatment of spontaneously diabetes-prone NOD mice with sIFNγR or anti-IFNγ AN-18 mAb prevented IDDM through generation of suppressor cells. To prove this, we used the accelerated form of diabetes inducible in newborn NOD mice by transferring splenic lymphoid cells from acutely diabetic NOD mice (24). In this study, newborn NOD mice were preinjected iv with splenic lymphoid cells (3×10^7) from euglycemic sIFNγ-treated NOD mice 12 h before the iv injection of spleen cells (3×10^7) from acutely diabetic female NOD mice. Two control groups were considered. One consisted of newborn NOD mice preinjected under the same experimental conditions with splenic lymphoid cells from 19-week-old euglycemic, PBS-treated, female NOD mice before transfer of spleen cells from acutely diabetic mice; in the other control group, newborn mice received only splenic lymphoid cells from acutely diabetic female NOD mice.

Measurement of humoral immune response to sIFNγR

The occurrence of anti-sIFNγR antibody was evaluated by solid phase ELISA as described previously (25). Microtiter plates were coated with sIFNγR in PBS. Different dilutions of serum samples from individual

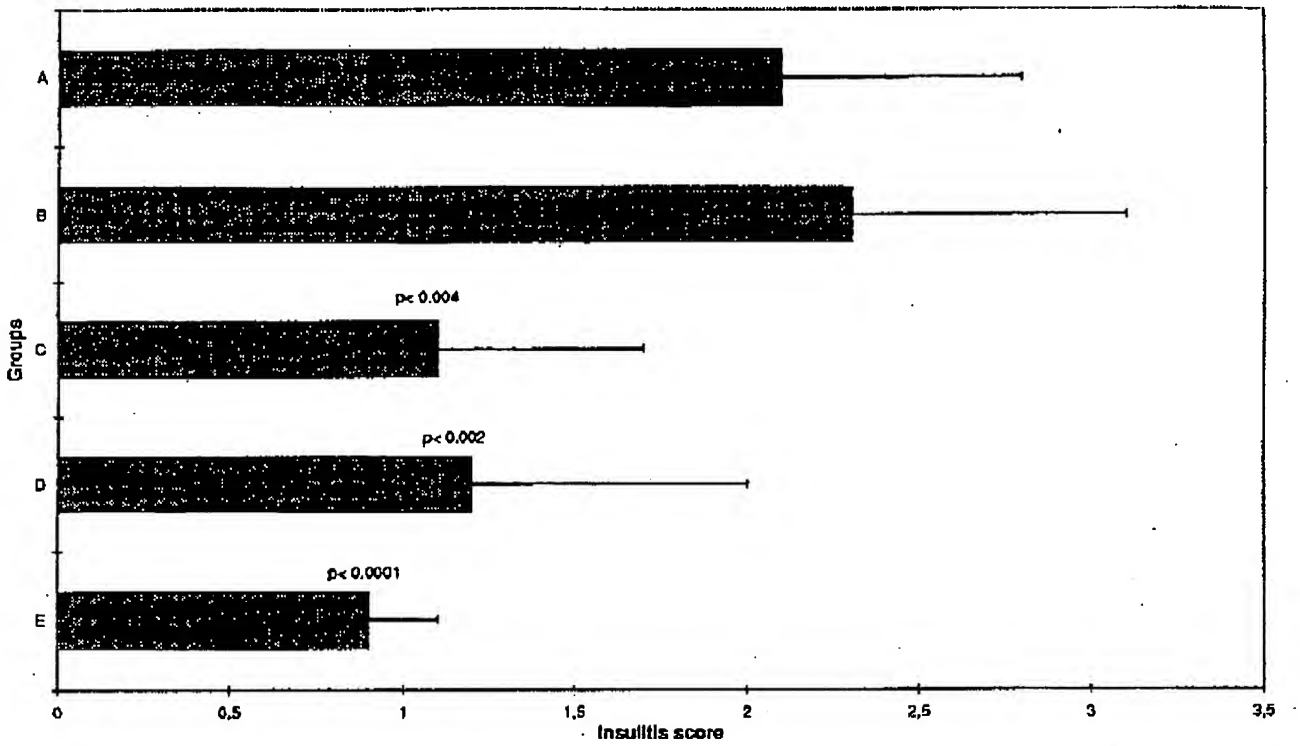

FIG. 1. Protection from insulitis development in NOD mice by early prophylactic treatment with XMG 1.2 and AN-18 anti-IFNγ mAbs or sIFNγR. Euglycaemic 4-week-old female NOD received for 9 consecutive weeks mouse anti-IFNγ mAbs, XMG1.2 (group C; 100 μg, twice a week, ip) or AN-18 (group D; 250 μg, twice a week, ip), or sIFNγR (group E; 250 μg/day, six times a week, ip). Control mice were treated with either PBS (group A 200 μl, 6 times a week, ip) or irrelevant rat IgG (group B; 250 μg, twice a week, ip). At 18 weeks of age, the mice were killed, and pancreata specimens were collected for histological analysis. For statistical analysis, group E was compared to group A, and groups C and D were compared to group A. Each group contained eight mice. Data are the mean ± SD.

mice treated with either sIFNγR or PBS were incubated for 2 h at room temperature. After extensive washing, a 1:1500 dilution of an alkaline-phosphatase-conjugated sheep antimouse Ig was used as the second step reagent.

Measurement of the immune responses to XMG1.2 and AN-18 mAbs

The formation of murine antibodies to XMG 1.2 and AN-18 was studied by a solid phase ELISA previously described by Williams *et al.* (26). In brief, microtiter plates, coated for 1 h at room temperature with 5 μg/ml of either XMG 1.2 or AN-18, were blocked and then incubated with serially diluted sera. After washing, horseradish peroxidase-conjugated goat antimouse IgG was used as the second antibody at a 1:3000 dilution in 2% bovine gamma globulin (BGG) and 1% BSA (75 μl/well) and kept at room temperature for 1 h. After washing, the colorimetrical reaction was developed by adding *o*-phenylenediamine in PBS for 10 min. Readings were performed at 490 nm (Titertek, Flow Labs, Rockville, MD) after stopping the reaction with 2 N H_2SO_4. Results are expressed as mean OD values.

Measurement of unbound XMG1.2 mAb

Microtiter plates were coated with recombinant murine IFNγ (10 μg/ml), blocked, and then incubated with test sera. Goat antirat IgG-alkaline phosphatase conjugate was added, followed by substrate addition. Quantitation was obtained by reference to a sample of a known concentration of XMG 1.2. Results are expressed (micrograms per ml) as the mean (±SD) unbound XMG 1.2.

Histological examination of pancreatic islets

Histological examination of pancreatic islets was performed in a blind fashion by 2 pathologists unaware of the status and/or the treatment of the animals, as described previously (27). At least 10 islets were counted for each pancreas. The degree of mononuclear cell infiltration was graded as follows: 0, no infiltrate; 1, periductular infiltrate; 2, periislet infiltrate; 3, intraislet infiltrate; and 4, intraislet infiltrate associated with β-cell destruction. The mean score for each pancreas was calculated by dividing the total score by the numbers of islets examined.

Statistics

Results are expressed as the mean ± SD. Data were analyzed using ANOVA. In situations considered significant, as appropriate, the *post-hoc* least significance difference was used to determine where pairwise differences existed. The incidence of diabetes among different groups was tested for significance using the χ^2 test with Yates' correction. $P < 0.05$ was considered significant.

Results

Spontaneous IDDM

Upon early prophylactic treatment, sIFNγR prevents insulitis development in NOD mice even more efficiently than XMG 1.2 anti-IFNγ mAb, and it exerts more powerful immunomodulatory effects on cytokine secretion than XMG 1.2 mAb.

TABLE 1. Experimental design and effects of prophylactic treatment with PBS, rat IgG, AN-18 or XMG 1.2 anti-IFNγ mAb, and m sIFNγR on the development of spontaneous diabetes and insulitis in NOD mice

Groups	No. of subjects	Treatment (μg)	Diabetes incidence (%)	Insulitis scores
F	20	PBS	14/20 (70)	2.9 ± 0.8
G	18	Rat IgG (200)	13/18 (72.2)	3.1 ± 0.7
H	21	XMG 1.2 (200)	3/21 (14.2)[a]	2.6 ± 0.9
I	21	AN-18 (500)	4/21 (19)[b]	2.7 ± 0.8
J	11	mIFNγR (400)	2/11 (18.2)[c]	2.9 ± 0.6

Sixteen-week-old euglycemic female NOD mice were treated for 8 consecutive weeks with two injections per week of rat IgG, AN-18, or XMG 1.2 or with three injections of PBS or sIFNγR. All treatments were given ip. For statistical analysis, groups H and I are compared to group F, and group J is compared to group F. Insulitis scores are shown as the mean ± SD. Significance was determined by χ^2 test with Yates' correction.

[a] $P < 0.0008$ *vs.* group G.
[b] $P < 0.002$ *vs.* group G.
[c] $P < 0.02$ *vs.* group F.

As expected, clear signs of early insulitis with peri-intraislet infiltration of the β-cells were observed at the age of 13 weeks in most control NOD mice treated with either PBS or irrelevant rat IgG (Fig. 1). In contrast, although the incidence of insulitis in the mice treated with XMG 1.2, AN-18, or sIFNγR was not different from that observed in control mice (data not shown), the process was significantly milder in the mice that received either of the two anti-IFNγ mAbs and to an even greater extent in those treated with sIFNγR (Fig. 1).

Along with the development of spontaneous diabetes, NOD mice also exhibit an altered pattern of cytokine production *in vitro*, which might be implicated in the pathogenesis of the disease, with augmented secretion of IFNγ and decreased release of IL-2, IL-4, and TNF-α (see Ref. 28 for a review). Thus, we wondered whether the antidiabetogenic action of the IFNγ inhibitors could be related to a change in the cytokine secretory capacity of NOD mice.

As previously observed for the histological analysis, sIFNγR exhibited a more powerful modulatory effect than XMG1.2 mAb on cytokine secretion, which resulted in a significant reduction of Con A-induced IL-2 secretion and an augmented release of both unstimulated and LPS-induced IL-6. Hence, when Con A-induced, T cell-derived cytokines were considered, the only significant effect we noticed was the marked reduction of IL-2 secretion in the mice treated with sIFNγR compared to the PBS-treated control group (Fig. 2). A trend toward decreased release of IL-2 was also observed in the mice treated with XMG 1.2 mAb, but the effect was not statistically significant (Fig. 2). No significant change was observed among the three groups considered in the Con A-induced secretion of IFNγ and IL-6 (Figs. 2 and 3). Con A-induced IL-4 and IL-10 secretion was always below the limit of sensitivity of the assays. In the same manner, unstimulated supernatants contained no detectable amounts of IL-2, IL-4, IL-10, and IFNγ.

On the other hand, when LPS-induced, and thus primarily macrophage- and B lymphocyte-derived, cytokines were measured, we found that SLC obtained from both XMG 1.2- and sIFNγR-treated groups secreted significantly larger amounts of IL-6 than those obtained from the PBS-treated control group. Here again, the effect was more evident for sIFNγR, which, unlike XMG1.2, could significantly increase the spontaneous release of IL-6 compared to that in PBS-treated controls (Fig. 3). No effects of either XMG 1.2 or sIFNγR on the Con A- or LPS-induced secretion of IL-10 and TNF-α could be determined, because the contents of these cytokines in cell supernatants were in each case below the limit of sensitivity of the assay we used. This was confirmed using two different ELISA kits for each cytokine (Biosource and Endogen; data not shown).

TABLE 2. Experimental design and effects of the treatment with PBS, rat IgG, AN-18 or XMG 1.2 anti-IFNγ mAb, and m sIFNγR on the development of CY-induced diabetes and insulitis in NOD mice

Groups	No. of subjects	Treatment (mg)	Diabetes incidence (%)	Insulitis scores
K	19	PBS	13/19 (68.4)	3.2 ± 0.6
L	19	Rat IgG (1)	6/13 (46.1)	2.1 ± 0.6
M	18	XMG 1.2 (1)	1/18 (5.6)[a]	1.8 ± 0.9
N	13	AN-18 (0.5)	5/13 (38.4)	2.9 ± 0.8
O	20	AN-18 (1.5)	2/21 (9.5)[b]	2.7 ± 0.9
P	13	m sIFNγR (0.25)	4/13 (30.7)	2.7 ± 0.8
Q	9	m sIFNγR (0.4)	5/9 (55.6)	2.6 ± 0.8

One hundred to 120 old euglycaemic female NOD mice were challenged with CY (300 mg/kg) on day 0. Treatment was given daily from days −2 to 13 in groups K and P and on alternate days in group Q; the mice from the other groups were treated on days −2, 3, 6, and 9. All injections were given ip. For statistical analysis, groups P and Q are compared to group K, and groups M, N, and O are compared to group L. Insulitis scores are shown as the mean ± SD. Significance was determined by chi-square with Yates' correction.

[a] $P < 0.03$.
[b] $P < 0.04$.

Late prophylactic treatment with sIFNγR is as effective as XMG 1.2 anti-IFNγ mAbs in preventing IDDM development in NOD mice. As expected, IDDM developed in the majority of the NOD mice that had been treated with either PBS or irrelevant rat IgG for 8 consecutive weeks and served as control groups. In contrast, the incidence of the disease was substantially reduced in the three groups of mice treated with sIFNγR, XMG 1.2, or AN-18 (Table 1). However, at least for sIFNγR and AN-18, continuous administration seemed to be necessary for the antidiabetogenic effect to be maintained. Thus, animals from these 2 groups developed IDDM with similar incidence and comparable kinetics after treatment withdrawal, with 3 of 5 and 5 of 10 of the mice that had been treated with sIFNγR or AN-18, respectively, becoming hyperglycemic between the 5th and 10th weeks of the follow-up period.

A severe insulitis process also occurred in the two control groups of NOD mice regardless of their clinical status (Table 1 and data not shown). In contrast to the high efficiency with which each of the three IFNγ inhibitors blocked the clinical outcome of diabetes, neither of them offered clear protection from the development of insulitis. So, although NOD mice treated with XMG 1.2, or AN-18 exhibited a trend toward less severe insulitis compared to both PBS- and rat IgG-treated control mice, the difference was not statistically significant (Table 1).

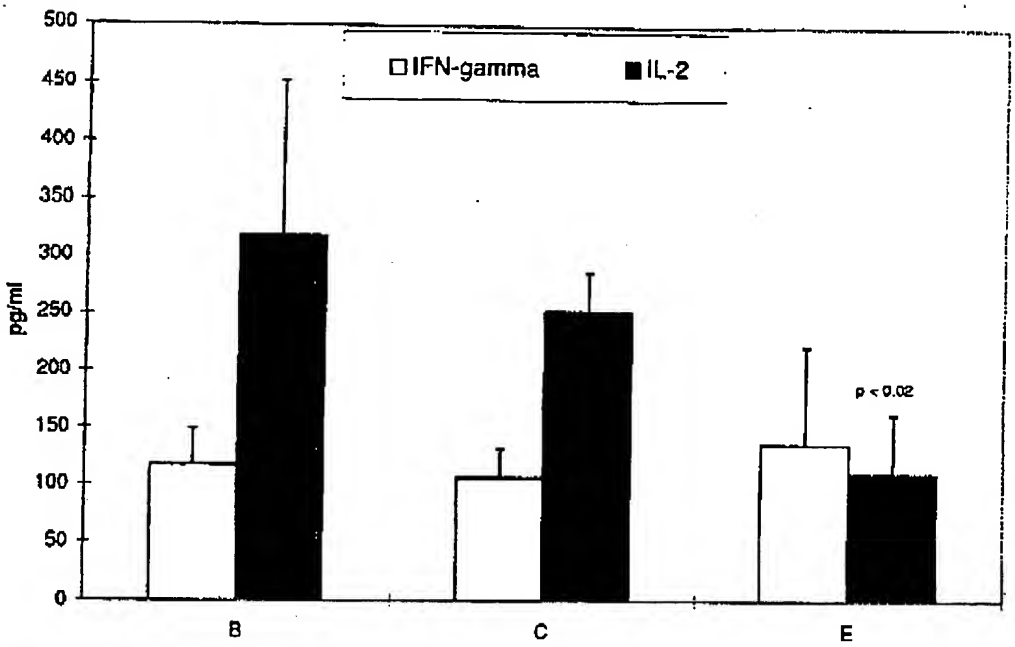

FIG. 2. *Ex vivo* effects of XMG 1.2 anti-IFNγ mAbs and sIFNγR on Con A-induced secretion of IL-2 and IFNγ. After 9 weeks of continuous treatment with irrelevant rat IgG (group B), XMG1.2 anti-IFNγ mAb (group C), or sIFNγR (group E), euglycemic NOD mice were killed, and individual spleens were collected. Splenic lymphoid cells were cultured for 48 h at 37 C in 5% CO_2 in the presence of Con A (4 μg). The cytokine content of each culture supernatant was determined by ELISA. For statistical analysis, each group was compared to group B. Data are shown as the mean ± SD.

CY-induced IDDM

Both XMG 1.2 and AN-18 anti-IFNγ mAbs, but not sIFNγR, suppress CY-induced diabetes in NOD mice. An accelerated form of autoimmune diabetes may be provoked in NOD mice by injecting them with one or two large doses (200–350 mg/kg daily) of CY at 100–120 days of age. CY seems to act by inhibiting suppressor cell function (29–31), thus allowing autoreactive cells to cause complete β-cell destruction and diabetes within 14–28 days after the first challenge. IFNγ plays a central pathogenic role in this model (4, 5), and its release seems to occur in a more vigorous and abundant fashion than in the spontaneous form, as judged by *ex vivo* studies in which β-cells infiltrating lymphocytes secrete IFNγ shortly after CY challenge (4). The requirement for much larger doses of anti-IFNγ mAbs necessary for the prevention of CY-induced diabetes compared to spontaneous diabetes (4–6) (see below) further substantiates this experimental observation.

In our study, the majority of control NOD mice treated with either PBS or rat IgG have developed an acute form of IDDM with glycosuria, hyperglycemia, and severe insulitis within 15 days after challenge with CY (Table 2). In contrast, confirming previous results (4, 5), treatment with anti-IFNγ mAb XMG 1.2 or AN-18 significantly suppressed the development of diabetes (Table 2). The dose dependency of the phenomenon for AN-18 was investigated, and the results showed that its preventive action is clearly dose dependent, with the effect achieving statistical significance at the highest doses.

Unexpectedly, in contrast to the results obtained in the spontaneous model, the antidiabetogenic action of sIFNγR in the CY-induced form of diabetes was weaker than that afforded by either XMG 1.2 or AN-18 anti-IFNγ mAbs. Thus, although a trend toward reduced incidence of diabetes vs. PBS-treated controls was noticed in the two groups of mice treated with sIFNγR, with the effect being more evident when sIFNγR was administered daily at the dose of 0.25 mg/mouse from 2 days before until 13 days after CY challenge (Table 2), the phenomenon was not statistically significant, possibly because of the smaller size of the experimental groups compared to that of the control group (Table 2).

Finally, neither the anti-IFNγ mAbs nor sIFNγR diminished the insulitis process in CY-challenged NOD mice, whose insulitis score was only slightly, not significantly, milder than that in PBS-treated controls (Table 2). No significant difference could be found in the insulitis score between CY-challenged NOD mice that did or did not develope IDDM (data not shown).

IDDM transfer studies: blockade of endogenous IFNγ with specific inhibitors does not protect NOD mice from diabetes development through generation of suppressor cells. By 45 days after the injection of spleen cells from acutely diabetic NOD mice, an acute form of IDDM occurred in six of six newborn NOD mice; a trend toward a reduced incidence of the disease was observed in the group of mice preinjected with spleen cells from sIFNγR-treated NOD mice, only three of five of which became diabetic. However, the effect did not depend on the

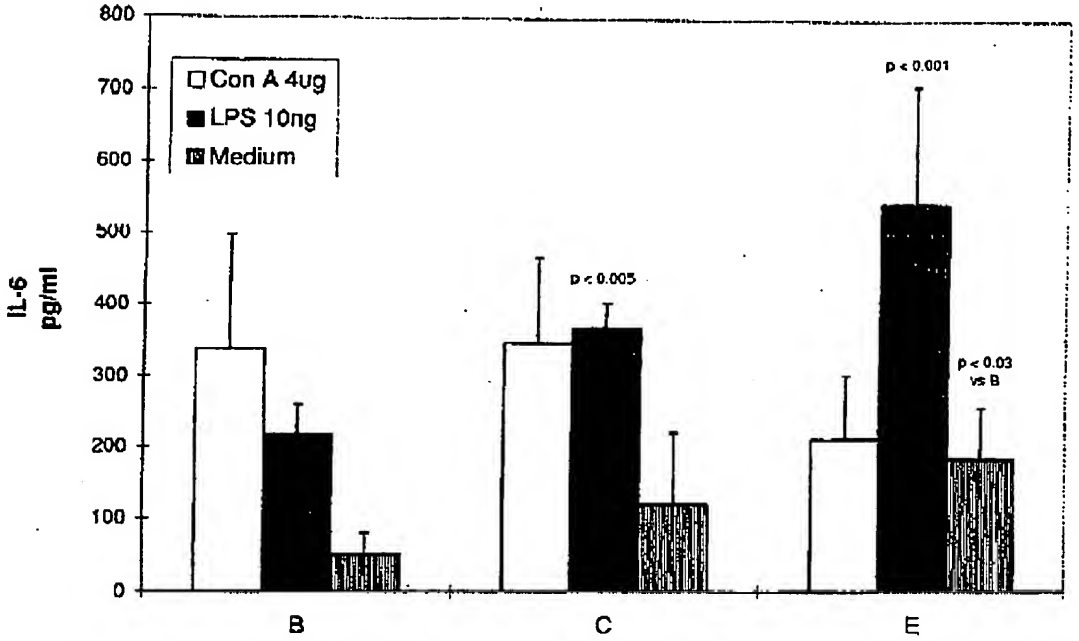

FIG. 3. *Ex vivo* effects of XMG 1.2 anti-IFNγ mAbs and sIFNγR on spontaneous, LPS-induced, and Con A-induced secretion of IL-6. After 9 weeks of continuous treatment with irrelevant rat IgG (group B), XMG1.2 anti-IFNγ mAb (group C) or sIFNγR (group E), euglycemic NOD mice were killed and individual spleens collected. Splenic lymphoid cells were cultured for 48 h at 37 C in 5% CO_2 in the presence or absence of LPS (10 ng) or Con A (4 μg). IL-6 was measured by ELISA. For statistical analysis, each group was compared to group B. Data are shown as the mean ± SD.

treatment received *in vivo*, as a reduced incidence of diabetes had also occurred in newborn NOD mice preinjected with spleen cells from PBS-treated NOD mice (four of seven).

Immunogenicity of sIFNγR, XMG 1.2, and AN-18: upon prolonged treatment, anti-IFNγ mAbs, but not sIFNγR, induce antibody production in NOD mice. As shown in Fig. 4, a significant increase in the blood titers of murine IgG directed against XMG 1.2 and AN-18 was noticed in the NOD mice that received these mAbs. This increase first became appreciable after 3 weeks of treatment and was maximal at 6 and 7 weeks when it reached a plateau that was maintained until the end of the treatment. Interestingly, measurement of the levels of unbound XMG1.2 in the circulation of the NOD mice treated with this mAb revealed a progressive decline of unbound mAb that well paralleled the development of anti-rat IgG (see Fig. 5).

In contrast, NOD mice treated with sIFNγR showed only a slight elevation of specific Ig antibody that, even though more pronounced at 5 and 6 weeks, was not statistically significant (Fig. 4).

Discussion

We demonstrated for the first time the efficacy of prolonged prophylactic treatment with sIFNγR in the prevention of histological and clinical signs of autoimmune diabetogenesis in NOD mice. Clinical, pathogenic, and immunopharmacological considerations worthy of attention may be drawn from our study.

The lack of immunogenicity of sIFNγR may have great relevance for the potential use of this IFNγ inhibitor in the management of human IDDM and possibly other autoimmune conditions. The biological relevance of antixenotypic antibody formation and its interference with anticytokine antibody therapy is emphasized by a recent study in which a rat antimouse IL-6 mAb is only effective in preventing SLE-like syndrome in (NZB×NZW)F1 mice if formation of antirat IgG by the mice is suppressed with a short course of tolerizing anti-CD4 mAb (32). In our study, although murine IgG directed against the two mAbs were produced from the mice treated for 8 weeks with either XMG 1.2 or AN-18, both of these mAbs protected NOD mice from diabetes development with a degree of protection comparable to that observed with the nonimmunogenic sIFNγR. This is probably due to the fact that a substantial increase in murine IgG directed against the two mAbs was first detectable in NOD mice 3 weeks after the first injection, a period within which endogenous IFNγ may have been sufficiently antagonized by the mAbs so as to afford temporary blockade of disease progression and delay its onset. On the other hand, not even by sIFNγR treatment would complete and long lasting neutralization of endogenous IFNγ be accomplished, because although it is not immunogenic, the short half-life it possesses (1 h after ip injection) (14) rapidly reduces its circulating levels and bioavailability.

Unexpectedly, when used in the CY-induced form of diabetes, sIFNγR offered only a slight and not significant preventive action, which contrasts with the clear-cut efficacy of anti-IFNγ mAbs, previously demonstrated by two indepen-

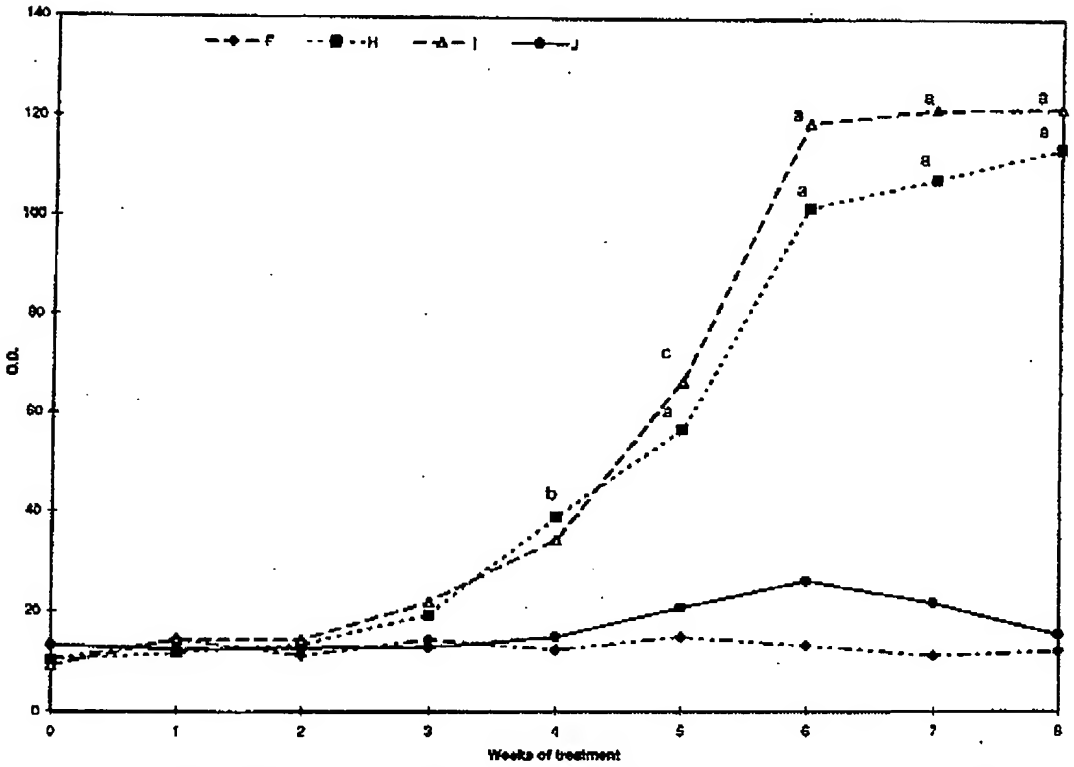

FIG. 4. Formation of murine IgG anti-XMG 1.2 and AN-18 mAbs in NOD mice. Sixteen-week-old NOD mice were bled before and weekly after beginning the treatment with PBS (F), XMG 1.2 (H) or AN-18 (I) anti-IFNγ mAb, or sIFNγR (J), and the production of antibody directed against either of the two mAbs or sIFNγR was assessed by solid phase ELISA. Data are representative of seven mice per group. One to 100 diluted sera are shown. Each group is compared to the pretreatment value. a, $P < 0.001$; b, $P < 0.005$; c, $P < 0.003$; d, $P < 0.01$; e, $P < 0.03$. SDs are within 60%, with a 32% mean value.

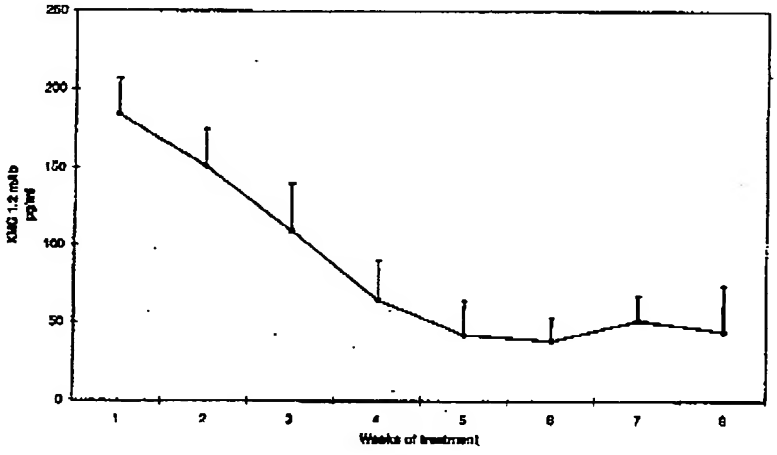

FIG. 5. Levels of unbound XMG1.2 mAb in NOD mice. Sixteen-week-old NOD mice were bled weekly after beginning treatment with XMG 1.2 anti-IFNγ mAb, and the levels of unbound concentrations of mAb antibody were assessed by ELISA. Data are shown as the mean ± SD.

dent studies (4, 5) and presently confirmed. The reason for this is not known. As previously mentioned, the possibility cannot be ruled out that sIFNγR has, in fact, prevented CY-induced diabetes, but the smaller size of the experimental groups *vs.* the number of PBS-treated control mice may have blunted statistical significance. Further studies are required to test this hypothesis with larger numbers of mice and possibly higher doses of sIFNγR. Nonetheless, the higher efficacy of the anti-IFNγ mAbs over the sIFNγR in CY-induced diabetes could be be due to the peculiar pathophysiology of diabetes in this model and the biological and pharmacokinetic properties of the different specific IFNγ

inhibitors used. Thus, the exuberant production of endogenous IFNγ known to be triggered by CY challenge (4) may have been sufficiently large to supersede the neutralizing capacity of sIFNγR, which may, in turn, have been minimized by its short persistency in the blood. On the other hand, these experimental conditions may have optimized the action of anti-IFNγ mAbs for both their long half-life (10–15 days) and the short frame time considered (15 days), within which, as inferred from the data obtained from the spontaneous model, neutralizing antibodies may have only minimally interfered with their action.

Although the development of insulitis could only be prevented in a clear-cut fashion when specific IFNγ inhibitors were administered early in the course of the disease, both sIFNγR and the anti-IFNγ mAbs successfully inhibited IDDM development in those NOD mice treated late during the prediabetic period and which exhibited a prevalence and extent of insulitis comparable to that of control mice. Although a discrepancy between histological and clinical processes was previously observed in NOD mice with other immunotherapeutical approaches (20, 33), this finding underscores the essential role of IFNγ in both the afferent and efferent phases of IDDM and shows that the entire diabetogenic process may be prevented if the cytokine is blocked early during disease development. However, the finding that specific IFNγ inhibitors can prevent IDDM development even when they are first administered at a late stage of the disease and in the presence of active insulitis can be important for the clinical setting, where prophylactic interventions can only be performed in those subjects exhibiting metabolic and immunological signs presumably associated with actively ongoing disease.

That anti-IFNγ mAbs were ineffective on the insulitis process in the CY model of diabetes contrasts with other studies in which the anti-IFNγ mAb (RA-642) drastically reduces the extent of insulitis in CY-treated NOD/Whei mice (4) and also prevents insulitis development in the adoptive transfer model of diabetes in NOD mice (5). Although the different experimental conditions used in this latter model make it difficult to compare the data, the histological discrepancy with the study by Campbell *et al.* (4) may depend on the different colonies of NOD mice, because at 100–120 days of age, the NOD/Lt mice we used exhibit a more severe insulitis than the NOD/Whei mice used by Campbel *et al.* (34). Thus, the capacity of anti-IFNγ mAb to successfully reduce insulitis in CY-challenged NOD/Whei mice may have been favored by the less advanced, and possibly reversible, phase of β-cell destruction occurring in this colony of NOD mice compared to the Lt counterpart. On the other hand, the inability of IFNγ inhibitors to reduce the development of insulitis when administered under a late prophylactic regimen accords with a study from Jacob *et al.*, in which the DB-1 anti-IFNγ mAb administered for 8 consecutive weeks to 7- to 8-week-old female NOD mice did not affect the progression of insulitis in these mice (35). Taken together, these results show variable effects of IFNγ inhibitors on the insulitic process in the NOD mouse, which may depend on the NOD colony considered, the potency and immunogenicity of the IFNγ inhibitor, and the ages of the mice when the treatment is started.

The recurrence of diabetes after treatment withdrawal and the inability of spleen cells from either anti-IFNγ- or sIFNγR-treated animals to protect syngeneic recipients from IDDM development both indicate that blockage of endogenous IFNγ neither eliminated autoreactive effectors nor induced suppressor cells. In contrast, in *ex vivo* conditions, SLC from the NOD mice that received either anti-IFNγ mAb or, in particular, sIFNγR produced less IL-2 and more IL-6 than SLC from control mice, suggesting a modulatory action of IFNγ inhibitors on the cytokine network. This action may have contributed to the beneficial effects, because IL-2 is pathogenically involved in IDDM in NOD mice (36). The possible significance of the up-regulated production of IL-6 is less clear, as this cytokine itself has been incriminated as a possible mediator of β-cell destruction (2, 4). However, IL-6 has been shown to exert powerful antiinflammatory properties both *in vitro* (37) and *in vivo* (38), and these latter effects could have prevailed under the experimental conditions considered. Interestingly, if the modulation of IL-2 and IL-6 production contributed to the antidiabetogenic action of the IFNγ inhibitors, the greater immunomodulatory effect of sIFNγR over XMG 1.2 mAb would then accord with the better histological protection offered by the sIFNγR. Although the reason for the greater efficiency of sIFNγR over XMG1.2 mAb is not known, the progressive reduction of the unbound levels of XMG1.2 observed in NOD mice upon prolonged treatment anticipates that this effect could depend on a better neutralization of endogenous IFNγ by sIFNγR during the last periods of the *in vivo* study.

The possibility of preventing spontaneous IDDM in NOD mice with sIFNγR may be important in the clinical setting, as the complementary DNA encoding the extracellular domain of the receptor was engineered and expressed in both *Escherichia coli* and the insect/baculovirus cell system as a soluble form of the receptor (39). At present, if these data may somehow mirror the impact of sIFNγR therapy in human IDDM, some points can be anticipated to warrant particular attention, including the above-mentioned reversibility of the antidiabetogenic action and the short half-life of this molecule. Certainly, the continuous and possibly life-long treatment with specific IFNγ inhibitors may not be clinically feasible, and therapeutic approaches based on short courses of treatment with multiple agents, including other proinflammatory cytokine inhibitors (sTNF-R, sIL-1R, and IL-1 receptor antagonist), antiinflammatory cytokines (IL-10, IL-13, and TGFβ), or subtherapeutic doses of cyclosporin A should be considered. Moreover, we have previously underlined how the disadvantage of sIFNγR over anti-IFNγ mAb therapy is the shorter half-life of the former. In the murine system we have overcome this problem by generating one mIFNγR-Ig fusion protein that is not immunogenic, possesses a 40 times longer half-life than sIFNγR, and is biologically active *in vivo* in the model of low dose streptozocin-induced diabetes (40). Along this line of reasoning, similar approaches could be used to generate human IFNγR-Ig fusion proteins.

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